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THE HYDROLYSIS PRODUCT OF ICRF-187 PROMOTES IRON-CATALYSED HYDROXYL RADICAL PRODUCTION VIA THE FENTON REACTION

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Abstract—*d*-1,2-Bis(3,5-dioxopiperazine-1-yl)propane (ICRF-187) (ADR-529) is a drug that ameliorates the cardiotoxicity of Adriamycin®. The drug enters cells where hydrolysis leads to its diacid diamide product, *dl*-*N*,*N*′-dicarboxamidomethyl-*N*,*N*′-dicarboxymethyl-1,2-diaminopropane (ICRF-198) (ADR-925), which is structurally similar to ethylenediaminetetraacetic acid (EDTA). The protective mechanism of ICRF-187 is unknown, but a plausible explanation is that ICRF-198 chelates iron intracellularly to prevent iron-dependent free radical reactions such as hydroxyl radical (·OH) production. We have compared Fe(ICRF-198) with Fe(EDTA) in its ability to promote ·OH formation in several Fenton reaction systems. The Fenton reaction was studied with H_2O_2 and Fe^{2+} chelates or catalytic amounts of the iron chelates in the presence of Adriamycin radicals, paraquat radicals, superoxide anion radicals ($O_2^{\cdot-}$), and ascorbate as reducing species. ·OH was detected with deoxyribose and dimethyl sulfoxide. The two methods gave comparable results. Fe(ICRF-198) was 80–100% as effective as Fe(EDTA) at promoting ·OH production in the presence of the organic radicals and ascorbate, 30–70% in the presence of $O_2^{\cdot-}$, and 150% with non-cycling Fe^{2+}. Fe(EDTA) is a more efficient catalyst of ·OH production than physiological chelates such as ADP, ATP and citrate. Therefore, by comparing previous work which examined physiological chelates and Fe(EDTA) with the present work, Fe(ICRF-198) appears to be a better ·OH catalyst than the physiological chelates. These results suggest that ICRF-198 generated *in vivo* from ICRF-187 would not protect against intracellular ·OH production. They also imply that ·OH production may not be as important in Adriamycin cardiotoxicity as other radical reactions, such as lipid peroxidation and thiol oxidation, that are inhibited by ICRF-198.

Iron-catalysed reactions are thought to play an important role in oxidative cellular injury. The reactivity of iron depends on its chelated form [1] and chelators such as desferrioxamine and phenanthroline have been shown in some situations to protect cells against iron-dependent oxidative insult [2, 3]. The mechanism of protection is thought to involve complexing of iron so that it is unable to participate in redox cycling reactions which have lethal consequences for the cell. A major mechanism of iron-mediated injury is hydroxyl radical (·OH†) production by the Fenton reaction.

$$Fe^{2+}\text{ (chelate)} + H_2O_2 \rightarrow Fe^{3+}\text{ (chelate)} + OH^- + {}^{\cdot}OH. \quad (1)$$

A reducing system is also required so that the iron can act catalytically. Ferrous chelates with ethylenediaminetetraacetic acid (EDTA), and with physiological compounds such as ADP, ATP and citrate are able to participate in the Fenton reaction [4, 5]. However, the overall efficiency of the reaction depends also on the reductive step, and varies significantly depending on the nature of the reductant [1, 6–8]. In general, Fe(EDTA) is superior to physiological chelates at catalysing ·OH production.

There is considerable interest in the therapeutic potential of chelators that may stop intracellular iron from participating in deleterious oxidative reactions. A compound of particular interest is *d*-1,2-bis(3,5-dioxopiperazine-1-yl)propane (ICRF-187) which ameliorates the cardiotoxicity of the anticancer agent Adriamycin® both in cancer patients [9, 10] and animals [11, 12]. Although the mechanism of cardiotoxicity is not fully understood, there is a substantial body of evidence supporting redox cycling and oxidative injury [13–15]. ·OHs are frequently implicated as the damaging species. The way in which pretreatment with ICRF-187 protects against cardiac damage is unknown. However, ICRF-187 is known to enter cells where it is hydrolysed to its diacid diamide, *dl*-*N*,*N*′-dicarboxyamidomethyl-*N*,*N*′-dicarboxymethyl-1,2-diaminopropane (ICRF-198) [16]. ICRF-198 (Fig. 1) is a strong chelator similar in structure to EDTA [17] with a high affinity constant for iron [18]. It is able to remove Fe^{3+} from Fe^{3+}–Adriamycin complexes [17] and inhibits iron-dependent lipid peroxidation [19–21] and thiol oxidation [19] in microsomes and platelet membranes. Evidence from an isolated rat heart model suggests that Adriamycin-dependent ·OH production may be inhibited by ICRF-187 [22].

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† Abbreviations: DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ·OH hydroxyl radical; ICRF-187, *d*-1,2-bis(3,5-dioxopiperazine-1-yl)propane; ICRF-198, *dl*-*N*,*N*′-dicarboxamidomethyl-*N*,*N*′-dicarboxymethyl-1,2-diaminopropane; $O_2^{\cdot-}$, superoxide anion radical; TBA, thiobarbituric acid.

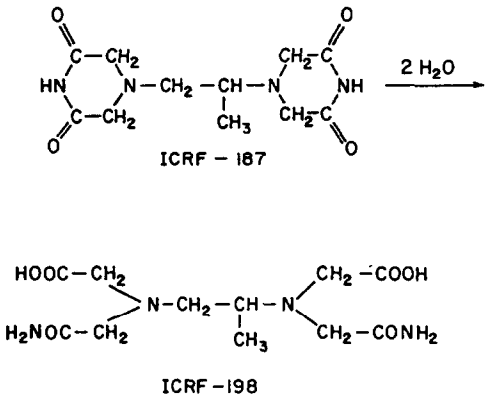

Fig. 1. Molecular structures of ICRF-187 and ICRF-198.

However, whether ICRF-198 can inhibit iron-dependent 'OH production has not been investigated directly.

If ICRF-187 protects cardiac cells from Adriamycin-induced damage by chelating intracellular iron, it could possibly be an effective therapeutic agent in other pathologies, e.g. paraquat toxicity and ischemia/reperfusion injury, that are thought to involve iron-dependent free radical reactions. Therefore, the ability of the drug to inhibit 'OH production needs to be assessed not only with Adriamycin but with other systems as well. In this work we examined the efficiency of Fe(ICRF-198) as compared to Fe(EDTA) to produce 'OH via the Fenton reaction. We have investigated the direct reaction between the ferrous chelates and H_2O_2 without any recycling of the iron, and in systems containing catalytic levels of iron which was reduced and recycled by superoxide anion radicals (O_2^-), paraquat radicals, Adriamycin radicals and ascorbate. The radicals were generated enzymatically by xanthine oxidase. Two *in vitro* methods were used to detect the Fenton oxidant. These were deoxyribose oxidation to thiobarbituric acid reactive substances an assay that has been extensively employed to detect 'OH [5–7, 23] and dimethyl sulfoxide (DMSO) oxidation to the stable primary product, methane sulfinic acid, which is detected by reaction with diazonium salts [24]. These two methods gave comparable results and show that, in most cases, Fe(ICRF-198) catalyses 'OH production with near equivalent efficiency to Fe(EDTA).

MATERIALS AND METHODS

Biochemicals were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) or from BDH Chemicals Ltd (Poole, U.K.) except for Adriamycin which was purchased from Farmitalia Carlo Erba Ltd and chelex resin from Biorad Laboratories (Richmond, CA, U.S.A.). ICRF-198 (ADR-925) was provided as a gift from Adria Laboratories (Dublin, OH, U.S.A.).

Experiments were carried out in 10 mM sodium phosphate buffer pH 7.4 treated with chelex resin. Glassware was acid washed to reduce iron contamination. Xanthine oxidase was mixed with EDTA in phosphate buffer and passed through a G-25 sephadex column also to minimize iron content [25]. All reactions were carried out in 10 mL tubes with a total volume of 1 mL. When reactions were run under N_2, solutions were bubbled with O_2-free N_2 for 2–5 min and sealed with rubber stoppers. The final reagent was added through the stopper with a syringe to start the reaction. Iron chelates were prepared by premixing $FeSO_4$ in equimolar HCl with either EDTA or ICRF-198. If oxygen is present at this stage, Fe^{2+} rapidly oxidizes. Therefore, in the enzyme systems and with ascorbate, the chelates would have been in their ferric forms at the start of the experiment. For the non-cycling Fe^{2+} experiments $FeSO_4$ and chelator were premixed under N_2 to maintain the ferrous chelate. Enzymatic reaction mixtures contained, at final concentrations, $FeSO_4$ (2 μM), chelator (40 μM), hypoxanthine (100 μM), either deoxyribose (5 mM) or DMSO (700 mM), hydrogen peroxide (200 μM), and either Adriamycin (32 μM), paraquat (0.5 mM) or no further addition. Adriamycin and paraquat radicals were generated under N_2 to avoid reaction with oxygen to give O_2^- [25, 26]. Superoxide was generated under air. Xanthine oxidase activity was measured as superoxide dismutase inhibitable cytochrome *c* reduction under air and approx. 0.004 U/mL were added to start the reaction. Reaction mixtures with ascorbate as the iron reductant contained $FeSO_4$ (2 μM), chelator (40 μM), either deoxyribose (5 mM) or DMSO (700 mM), and hydrogen peroxide (100 μM). Reactions were performed under air and started by the addition of ascorbic acid (100 μM). Reaction mixtures with non-cycling iron contained $FeSO_4$ (20 μM), chelator (40 μM), either deoxyribose (5 mM) or DMSO (700 mM), and hydrogen peroxide (50 μM). Solutions were bubbled with N_2 before adding the iron chelate mixture to start the reaction. All reaction mixtures were incubated at 37° for 30 min.

Deoxyribose oxidation was measured as thiobarbituric acid (TBA) reactive products at 532 nm [23] after incubation at 100° for 10 min with 0.5 mL trichloroacetic acid (2.8 g/100 mL) and 0.5 mL TBA (1 g/100 mL 0.05 M NaOH). Blanks contained all reagents except xanthine oxidase, ascorbate or the non-cycling Fe^{2+} (chelate).

DMSO oxidation to methane sulfinic acid was measured by adding the diazonium salt Fast Blue BB and measuring A_{425} as described by Babbs and Griffin [24]. Briefly, the 1 mL reaction mixture was adjusted to pH 2.5 with HCl, 0.5 mL of 30 mM Fast Blue BB was added, and the mixture was incubated at room temperature in the dark for 10 min. The colored diazosulfone product was extracted with 1 mL toluene:butanol (3:1), washed with 2.5 mL butanol-saturated water to remove any unreacted salt, and the color stabilized with 0.25 mL pyridine:acetic acid (95:5). Blanks contained all reagents except xanthine oxidase, ascorbate or the non-cycling Fe^{2+} (chelate).

RESULTS

OH production from five different Fenton systems

Table 1. Product yields of deoxyribose oxidation from five Fenton reaction systems with ICRF-198 or EDTA chelated iron

Fenton system	A_{532} Fe(EDTA)	A_{532} Fe(ICRF-198)	Product yield ratio ICRF-198:EDTA
Paraquat	1.98 ± 0.11	1.71 ± 0.08	0.86 ± 0.02
Adriamycin	0.52 ± 0.09	0.43 ± 0.07	0.84 ± 0.09
Superoxide	1.15 ± 0.10	0.30 ± 0.04	0.26 ± 0.02
Ascorbate	1.01 ± 0.02	0.90 ± 0.03	0.89 ± 0.03
Non-cycling Fe^{2+}	0.12 ± 0.01	0.18 ± 0.02	1.52 ± 0.13

Values are means ± SD of five sets of duplicates. The means and SDs of the product yield ratios were calculated from the ratios obtained in the individual experiments.

Experiments with paraquat, Adriamycin and non-cycling iron were carried out under N_2. Paraquat, Adriamycin and superoxide radicals were generated with xanthine oxidase and hypoxanthine as described in the Materials and Methods. Xanthine oxidase was added to the reaction mixture to give a rate of 3.17 ± 0.20 μM cytochrome *c* reduced/min under air. Radical generation rates under N_2 in the paraquat and Adriamycin systems are similar but not identical to this.

Blanks contained all reagents except xanthine oxidase, ascorbate or the non-cycling Fe^{2+} (chelate).

was measured. Fe^{2+} (ICRF-198) was compared with Fe^{2+} (EDTA) in the direct reaction with H_2O_2, and in systems where the Fe^{3+} (chelate) generated in Reaction 1 was recycled with ascorbate, O_2^-, Adriamycin radicals and paraquat radicals. The radicals were generated with xanthine oxidase, under air for O_2^- and under N_2 for Adriamycin and paraquat [7].

The optimal H_2O_2 concentration was evaluated for the xanthine oxidase experiments using the deoxyribose assay. Maximum TBA-reactive product yields were obtained with H_2O_2 concentrations of 100, 200 and 250 μM for the Adriamycin, superoxide and paraquat experiments, respectively (data not shown). In each radical generating system, the product yield ratio for Fe(ICRF-198):Fe(EDTA) remained constant between 100 and 250 μM H_2O_2. An H_2O_2 concentration of 200 μM was used for the remainder of the study.

The product yields of deoxyribose oxidation obtained with ICRF-198 and EDTA for the various Fenton reaction systems are shown in Table 1. Fe(ICRF-198) catalysed deoxyribose oxidation with similar efficiency to Fe(EDTA) when ascorbate or the organic radicals, paraquat and Adriamycin, provided reducing equivalents. Superoxide radicals, however, were less efficient at driving deoxyribose oxidation with Fe(ICRF-198), giving a product yield ratio of 0.26. When higher concentrations of the ferrous chelates were used without recycling, there was approximately 50% more deoxyribose oxidation with Fe^{2+} (ICRF-198) than with Fe^{2+} (EDTA).

Similar experiments were performed using DMSO to trap the Fenton oxidant. Yields of the DMSO oxidation product, methane sulfinic acid, were measured at 425 nm with representative absorbance spectra taken from 340 to 520 nm to verify the A_{425} peak (data not shown). Our spectra with all five systems were consistent with those reported previously for a xanthine oxidase/xanthine/Fe(EDTA) system [24]. In agreement with previous results [24], no sulfinate product was observed in the absence of reduced iron chelate, or in the recycling systems if xanthine oxidase or ascorbate was omitted from the reaction mixture. The yields of methane sulfinic acid obtained with ICRF-198 or EDTA in the various Fenton systems are shown in Table 2. Both iron chelates were nearly equally efficient catalysts of oxidation when paraquat radicals, Adriamycin radicals or ascorbate were used as reductants, giving product yield ratios which near unity. Consistent with data from the deoxyribose assay, O_2^- was less effective at driving Fe(ICRF-198)-catalysed DMSO oxidation as compared to Fe(EDTA). However, the ratio was not as low as observed for deoxyribose oxidation. Fe(ICRF-198) gave more DMSO oxidation as compared to Fe(EDTA) when the ferrous chelate was used without a reductant.

Both the deoxyribose and DMSO assays are used commonly to detect ·OH. However, it is recognized that a ferryl or equivalent species with reactivity different from ·OH can be produced in the Fenton reaction with some forms of iron [27, 28]. To investigate the reactivity of the oxidant produced with Fe(ICRF-198), scavenger studies were performed using the deoxyribose assay and xanthine oxidase-generated paraquat and Adriamycin radicals. As shown in Table 3, deoxyribose oxidation was inhibited by scavengers with the following order of potency: benzoate > formate > mannitol. Although the scavenging ability of mannitol in the Adriamycin system is surprisingly low, in general these results are similar to what has been observed with Fe(EDTA) in several Fenton systems [7, 29] and are as expected for competition for ·OH.

DISCUSSION

The protection given by ICRF-187 against Adriamycin cardiotoxicity and the inhibitory effect of its hydrolysis product, ICRF-198, against

Table 2. Product yields of DMSO oxidation from five Fenton reaction systems with ICRF-198 or EDTA chelated iron

Fenton system	A_{425} Fe(EDTA)	A_{425} Fe(ICRF-198)	Product yield ratio ICRF-198:EDTA
Paraquat	0.48 ± 0.08	0.46 ± 0.06	0.96 ± 0.09
Adriamycin	0.12 ± 0.03	0.10 ± 0.03	0.84 ± 0.09
Superoxide	0.18 ± 0.01	0.13 ± 0.01	0.71 ± 0.03
Ascorbate	0.70 ± 0.03	0.72 ± 0.03	1.03 ± 0.08
Non-cycling Fe^{2+}	0.09 ± 0.01	0.13 ± 0.02	1.49 ± 0.06

Values are means ± SDs of three to four sets of duplicates. The means and SDs of the product yield were calculated from the ratios obtained in the individual experiments.

Experiments with paraquat, Adriamycin and non-cycling iron were carried out under N_2. Paraquat, Adriamycin and superoxide radicals were generated with xanthine oxidase and hypoxanthine as described in the Materials and Methods. Xanthine oxidase was added to the reaction mixture to give a rate of 3.27 ± 0.18 μM cytochrome *c* reduced/min under air. Radical generation rates under N_2 in the paraquat and Adriamycin systems are similar but not identical to this.

Blanks contained all reagents except xanthine oxidase, ascorbate or the non-cycling Fe^{2+} (chelate).

Table 3. Effects of the hydroxyl radical scavengers, benzoate, formate and mannitol, on deoxyribose oxidation via the Fenton reaction with ICRF-198 chelated iron

Fenton system	Percent inhibition of TBA-reactive product formation: Benzoate	Formate	Mannitol
Paraquat	90.5 ± 0.5	75.5 ± 0.5	52.0 ± 4.0
Adriamycin	92.0 ± 4.0	69.0 ± 0.0	5.5 ± 5.5

Values are means of two sets of duplicates ± ranges.

Paraquat and Adriamycin radicals were generated with xanthine oxidase and hypoxanthine under N_2 as described in Materials and Methods. Reaction conditions were as in Table 1 with the following modifications to final concentrations.

Adriamycin studies: $FeSO_4$ (1 μM), ICRF-198 (10 μM), hypoxanthine (150 μM), hydrogen peroxide (100 μM), Adriamycin (30 μM).

Paraquat studies: $FeSO_4$ (2 μM), ICRF-198 (100 μM), hydrogen peroxide (250 μM) and paraquat (0.5 mM).

Scavenger concentrations were 20 mM.

various oxidative reactions [9–11, 19–21] suggest that Fe(ICRF-198) may be a poor catalyst of $^{\cdot}OH$ production. We have investigated this possibility, using two assays for $^{\cdot}OH$ to decrease the likelihood of the results depending on the method used. The relative product yield ratios observed for Fe(ICRF-198) and Fe(EDTA) in several Fenton systems are consistent between the two assays, and the scavenger profiles obtained with deoxyribose are consistent with the oxidant produced from Fe(ICRF-198) being $^{\cdot}OH$. Contrary to what might be expected, we found that in the direct reaction between the ferrous chelate and H_2O_2, Fe^{2+} (ICRF-198) was superior to Fe^{2+} (EDTA) at generating $^{\cdot}OH$. When catalytic amounts of iron chelate and a reducing system were used, the relative efficiency of the ICRF-198 chelate was decreased. This suggests that Fe^{3+} (ICRF-198) is not reduced as readily as Fe^{3+} (EDTA). However, with Adriamycin radicals, paraquat radicals and ascorbate, Fe(ICRF-198) was still nearly as efficient a catalyst as Fe(EDTA), and it was only with the O_2^- driven (Haber–Weiss) reaction that it gave a substantially lower $^{\cdot}OH$ yield. The ICRF-198 iron chelate, therefore, resembles the diethylenetriaminepentaacetic acid iron chelate in being a poor catalyst of the iron catalysed Haber–Weiss reaction [8, 30] while a good catalyst of other radical driven Fenton reactions [25, 26]. Ferrous complexes with ADP, ATP and citrate are capable of reacting with H_2O_2 to generate $^{\cdot}OH$ in reaction 1 [4, 5]. However, these potential physiological chelates are less efficient than Fe(EDTA) at catalysing paraquat and Adriamycin radical driven Fenton reactions and are very poor catalysts of the Haber–Weiss reaction [6–7]. Fe(ICRF-198), therefore, is a comparatively better catalyst of $^{\cdot}OH$ production than are the physiological chelates.

These results have a bearing on the mechanism by which ICRF-187 protects against Adriamycin-induced cardiotoxicity. Although several hypotheses have been proposed to explain this cardiotoxicity [31], substantial experimental evidence indicates that free radicals are involved [13–15]. A plausible, but not proven, mechanism of ICRF-187 action is via iron chelation. Reduction of Adriamycin by cellular enzyme systems leads to the Adriamycin semiquinone and consequent redox cycling. This can result in iron-dependent lipid peroxidation, thiol oxidation and $^{\cdot}OH$ production [32–34]. Our data indicate that Adriamycin radicals, and to a lesser extent O_2^-, are capable of driving the Fe(ICRF-198)-catalysed Fenton reaction to produce $^{\cdot}OH$ *in vitro*. In each case, when compared to physiological chelators such as ADP, ATP and citrate, Fe(ICRF-198) is the better catalyst. Although we are cautious about extrapolating our *in vitro* results, it appears that the putative generation of $^{\cdot}OH$ via the Fenton reaction

in vivo may not be minimized by ICRF-198 removing iron from nucleotide and citrate complexes in a physiological milieu. The observed protection by ICRF-187 against Adriamycin cardiotoxicity, therefore, may be evidence that 'OHs are not major contributors to the injury. This does not rule out a free radical mechanism, however. Adriamycin-dependent lipid peroxidation and oxidative inactivation of Ca-ATPase, which do not appear to involve free 'OH, are inhibited by ICRF-198 [19–21]. Other site specific reactions of iron bound to critical target molecules may also be inhibited.

Free radical injury is though to be important in paraquat toxicity and many other pathological processes. Although there is substantial evidence that iron-dependent reactions are important, the specific reactions involved are less clear. Whether catalytically active iron is available *in vivo* is uncertain. However, some evidence suggests that sufficient amounts are present to function in cytotoxic free radical processes [35]. Our results suggest that the general usefulness of ICRF-187 as a therapeutic agent against free radical injury would depend on whether or not 'OH is the major damaging species. ICRF-198, like EDTA, inhibits lipid peroxidation and inactivation of thiol enzymes in spite of promoting 'OH production by the Fenton reaction. If intracellular 'OH is singularly important in any toxicity, then iron chelation by ICRF-198 may not result in beneficial effects. If, however, other reactive species such as those that initiate lipid peroxidation are involved, then chelation by ICRF-198 may provide protection.

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